

Cost-Effective Microbiology Methods Rapid identification of Bacteria and Yeast

- I This presentation is based on the NCCLS (CLSI) document M35-A “Abbreviated Identification of Bacteria and Yeast; Approved Guideline.” In today’s environment of cost-containment, there is good reason to use abbreviated methods, provided that they give reliably accurate identifications.

References to Demonstrate Benefit

Doern, G., R. Vautour, M. Gaudet, and B. Levy. 1994. Clinical impact of rapid in vitro susceptibility testing and bacterial identification. J. Clin. Microbiol. 32:1757-1762.

MIC 9 h from +colony vs. 20 h- showed less mortality, length of stay and orders of laboratory tests

Barenfanger, J., C. Drake, and G. Kacich. 1999. Clinical and financial benefits of rapid bacterial identification and antimicrobial susceptibility testing. J. Clin. Microbiol. 37: 1415-1418.

Evaluated evening vs. next day - 5 h difference - length of stay and cost was significantly less

A. Pros and Cons of Rapid Testing.

Pros:

1. Minimal or less work than standard tests
2. Results out faster than by conventional methods (enhance patient care)
3. Often less expensive than standard tests

Cons:

1. Requires more technical expertise to be as accurate
2. Cannot be applied in all situations
3. May disrupt workflow

B. Criteria for Use of Rapid Tests

1. Only for certain organisms that can be identified by rapid methods
2. Errors will not have a negative impact on patient care
3. Sensitivity and specificity must be greater than 95%
4. Some organisms have “unique” reactions for easy, accurate identification –
5. Results are not “presumptive” and can be used for normally sterile body sites unless the user wishes to validate the result with additional tests.

“The identifications derived from the procedures outlined in this guideline are intended to be reported directly, not as “presumptive” identifications, since the likelihood of correct identification (>95%) is comparable to that of other conventional and automated methods.”

C. Factors to Keep in Mind

1. Methods are for colonies only - not intended for direct specimen testing
2. Only call the identification if all conditions are met by the isolate
3. Hold isolates your normal holding time (5-7 days) just in case

D. Technologist must...

1. Be sure colony is pure
2. Be able to recognize typical colony
3. Make accurate observations of reactions
4. Do not skip the Gram stain when required

E. Supervisor must...

1. Validate competency of staff to recognize colonies and reactions.
2. Be sure that all criteria of the identification are met.
3. Write clear procedures for tests
4. Assure that Quality Control meets local and federal guidelines.

CLIA 88:

All quality control activities must be documented.

(a) The laboratory must check positive and negative reactivity with control organisms--

(1) Each day of use for catalase, coagulase, beta-lactamase, and oxidase reagents and DNA probes;

(2) Each week of use for Gram and acid-fast stains, bacitracin, optochin, ONPG, X, and V discs or strips; and

(3) Each month of use for antisera....

(4) New lots/shipments of reagents, commercial tests prior to being used on patient specimens.

QC CLIA 2003: Bacteriology

All quality control activities must be documented.

(a) The laboratory must check positive and negative reactivity with control organisms:

(1) Each day of use for ~~catalase, coagulase, beta-lactamase, and oxidase reagents and DNA probes and beta-lactamase (except-cefinase)~~

(2) Each week of use for Gram and acid-fast stains, ~~bacitracin, optochin, ONPG, X, and V discs or strips; and~~

(3) Each ~~month~~ six months of use for antisera....

(4) New lots/shipments of reagents, commercial tests, etc. prior to being used on patient specimens, including catalase, coagulase, beta-lactamase (cefinase only)

Table 1. Organisms considered.

Organisms that are included in the document include most of the commonly isolated, clinically important aerobic bacteria and yeast and 14 common anaerobic organisms:

AEROBES

Escherichia coli
Haemophilus influenzae
Moraxella catarrhalis
Proteus mirabilis/penneri
P. vulgaris
Pseudomonas aeruginosa

Enterococcus species
Staphylococcus aureus
Streptococcus agalactiae
S. pneumoniae
S. pyogenes

YEASTS

Candida albicans
Cryptococcus neoformans
Candida (Torulopsis) glabrata

ANAEROBES

Bacteroides fragilis group
B. urealyticus
Bilophila wadsworthia
Clostridium difficile
C. perfringens
C. septicum
C. sordellii
C. tetani
Fusobacterium nucleatum
Peptostreptococcus spp
Porphyromonas spp.
Prevotella spp.
Propionibacterium acnes
Veillonella spp

II) GRAM-NEGATIVE ORGANISM IDENTIFICATION

A) *Escherichia coli*

IDENTIFICATION: Begin with indole and oxidase tests

1. GNR that are hemolytic on BAP; oxidase-negative and indole-positive are *E. coli*

a. Indole test:

Reagent: Either 5% *p*-dimethylaminobenzaldehyde or 1% paradimethylaminocinnamaldehyde in 10% (vol/vol) concentrated HCL

Procedure: Moisten filter paper with reagent. Using a wooden stick, rub portion of colony onto paper. Growth medium must contain an adequate amount of tryptophan and not contain dyes.

Interpretation: The development of a brown-red to purple-red color (benzaldehyde reagent) or blue color (cinnamaldehyde reagent) within 20 sec. indicates the presence of indole. Detectable indole will diffuse to colonies within 5 mm of a 2 to 3 mm colony, giving false-positive results.

b. Oxidase test:

Reagent: Aqueous solution of tetramethyl-*p*-phenylenediamine dihydrochloride.

Procedure: Pour onto filter paper. Using a wooden stick, rub portion of colony onto paper.

Interpretation: Colonies that give a positive reaction form a purple color within 10 sec of being rubbed onto the paper. Test separates *Enterobacteriaceae* from *Vibrionaceae* and pseudomonads.

2. Lactose-positive GNR (observed on EMB or MacConkey) that are not beta-hemolytic and are oxidase-negative and indole- positive and PYR-negative are *E. coli*.

PYR positive and negative reactions.

Some *Enterobacteriaceae* hydrolyze the substrate L-pyrrolidonyl β -naphthylamide (PYR), releasing β -naphthylamine which forms a fuchsia precipitate when aminocinnamaldehyde is added after 2 min. This test separates *E. coli* from *Citrobacter* and *Klebsiella oxytoca*, which are PYR +.

Table 2. PYR Reactions of common *Enterobacteriaceae*

Genus	Indole	PYR	Lactose
<i>Citrobacter</i>	Some +	+	V
<i>Enterobacter</i>	-	+	+
<i>E. coli</i>	+	-	V
<i>Serratia</i>	-	+	+
<i>Klebsiella</i>	Some +	+	+
<i>Yersinia</i>	Some +	+	- ⁺
<i>Morganella</i>	+	-	-
<i>Proteus</i>	Some +	-	-
<i>Providencia</i>	+	-	-
<i>Edwardsiella (beta hemolytic)</i>	+	-	-
<i>Shigella</i>	Some +	-	-
<i>Salmonella</i>	-	-	-

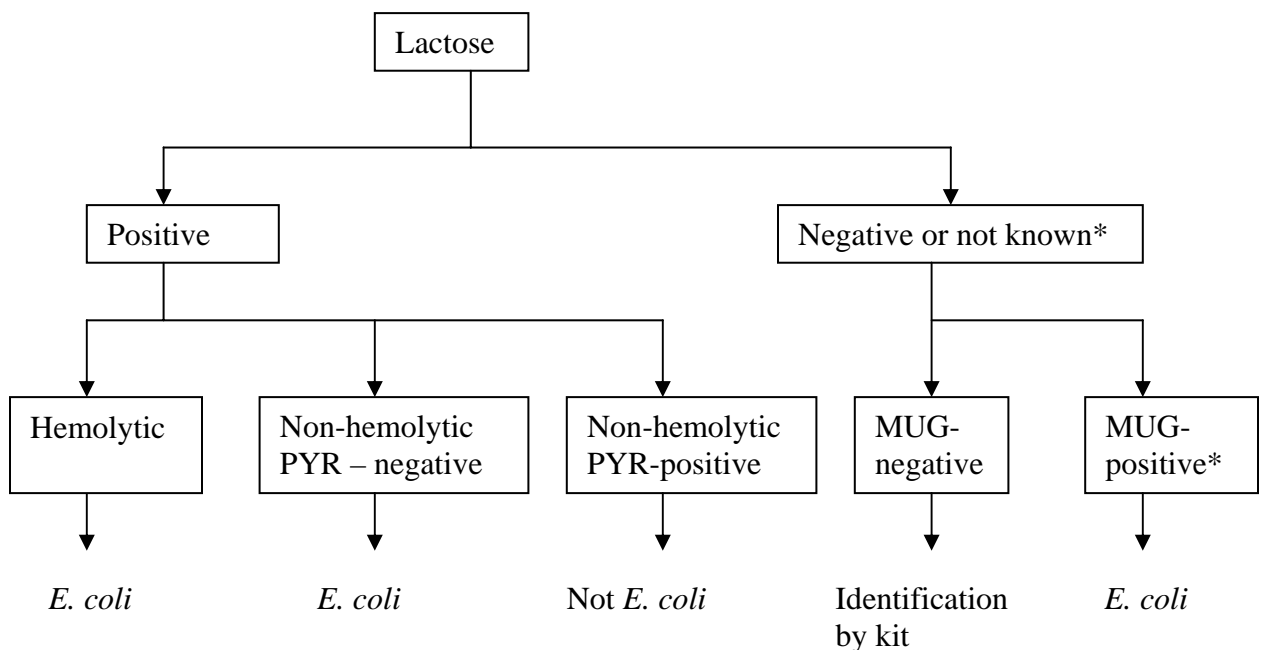
3. GNR that are oxidase-negative and indole-positive and MUG-positive are *E. coli* (Lactose lactose-negative or not known)

MUG test (reactions observed under ultraviolet light):

MUG (4-methylumbelliferyl- β -D-glucuronide) is hydrolyzed by *E. coli* to release a fluorescent dye. Most of the *Enterobacteriaceae* are MUG-negative and most *E. coli* are MUG-positive. *E. coli* 0157 is MUG-negative.

The MUG test is performed by inoculating a moistened disk with a colony and incubating the disk at 35°C for up to 2 h. Observe disks using a long-wave ultraviolet light. A positive reaction is indicated by an electric blue fluorescence. The tube MUG test can be done from MacConkey or EMB: Prepare by dissolving 0.5 g MUG into 100 mL M/15 Sorensen's buffer pH 7.5. Store in 0.25 ml amounts at -25°C in dark. See appendix for Sorensen's buffer recipe.

Figure 1. Algorithm for indole-positive, oxidase-negative identification.



***Limitations:**

1. Lactose-negative, beta-hemolytic colonies could be *Morganella morganii* or *Proteus vulgaris* in some geographic areas. *Edwardsiella tarda* is indole-positive and beta-hemolytic and is part of fecal flora. If these strains are not present in population being tested, the MUG test can be omitted on lactose-negative, beta-hemolytic colonies, from non-invasive sites.
2. A few stool pathogens are MUG-positive, so MUG should not be used to identify *E. coli* from the gastrointestinal tract. MUG can actually be used to rapidly screen for *E. coli* 0157 in stools; *E. coli* 0157 is MUG-negative.
3. Use care to pick colony on BAP that corresponds to colony on MAC or EMB.

Validation: In a 4 hospital laboratory study of over 1000 strains, the above algorithm was applied: 294 were identified as *E. coli* based on hemolysis, 628 were PYR-negative and lactose-positive, and 65 of the remaining 78 lactose-negative isolates were MUG-positive. The remaining 13 *E. coli* required conventional testing for identification. Of the 64 non-*E. coli* in the group of indole-positive strains, 3 were mis-identified as *E. coli*; a *Kluyvera*, *E. fergusonii* and *M. morganii*, for a sensitivity of 99.7%.

The net result of the study indicated that if the algorithm was applied, the cost savings would be \$3100 in reagents and 70 h in technologist time. Sensitivity so high that the algorithm could be used for definitive identification of all specimens, including invasively collected specimens.

Reference: York, M.K., E.J. Baron, M. Weinstein, R Thomson, and J.E. Clarridge. 2000. A Multi-Laboratory validation of rapid spot tests for identification of *Escherichia coli*. J Clin Microbiol 38: 3394-3398.

B) *Proteus* Identification

Table 3. Biochemical reactions of the *Proteus* species.

	Spreading on BAP (%)	Indole (%)	Ampicillin S (%)	ODC(%)	Maltose ferment (%)
<i>P. mirabilis</i>	96	2	95	99	0
<i>P. vulgaris</i>	64	98	0	0	97
<i>P. penneri</i>	65-90	0	0	0	100

Identification requirements for *Proteus*

Spreading: Indole-positive: → *Proteus vulgaris*
Indole-negative: → Ampicillin-susceptible: *Proteus mirabilis*
→ Ampicillin-resistant
→ Maltose-negative or Ornithine-positive: *Proteus mirabilis*
→ Maltose-positive or Ornithine-negative: *Proteus penneri*

Alternatively call isolate: *Proteus mirabilis/penneri*

C. Rapid Urea test will identify *Proteus*/*Morganella*.

Phenylalanine deaminase also tested with disk

Can be useful for identification of *Brucella*, *Helicobacter*, *Bordetella*, and *Corynebacterium*
Brucella:

Oxidase positive

Catalase positive

Urea positive

Gram-negative coccobacilli

D. Identification requirements for *Pseudomonas aeruginosa*

1. Oxidase-positive.
2. Grow on blood or chocolate agar as large colonies with metallic sheen, mucoid, rough, or pigmented and often beta-hemolytic. (On MacConkey, the colony appears as a non-lactose fermenter with green pigmentation, or metallic sheen).
3. A typical fruity or, grape-like odor.

Limitations: *Burkholderia cepacia* from cystic fibrosis (CF) patients may have fruity odor. Confirm colistin or polymyxin B susceptibility on all *Pseudomonas aeruginosa* from CF patients with disk, to avoid this pitfall. *B. cepacia* is resistant to these drugs. *Aeromonas* can appear metallic but can be ruled out by their positive indole reaction.

Table 4. Biochemical reactions among the *Haemophilus* species.

	V	REQUIRING	PORPHYRIN	LACT	UREA	IND	ODC	CAT
	(SATELLITE)							
<i>H. influenzae</i>	+	-	-	V	V	V	+	
<i>H. haemolyticus</i>	+	-	-	+	V	-	+	
<i>H. parahaemolyticus</i>	+	+	-	+	-	-	+	
<i>H. parainfluenzae</i>	+	+	-	V	V	V	V	
<i>H. paraphrophilus</i>	+	+	+	-	-	-	-	
<i>H. ducreyi</i>	-	-	-	-	-	-	-	

D. Identification requirements for *Haemophilus influenzae*

4. Gram negative coccobacillus or small rod
5. Growth as a large colony only on chocolate agar in 24 h **or** blood agar only around *Staphylococcus* streak in 5% CO₂ (V-factor)
6. Negative delta-aminolevulinic acid test (ALA test for porphyrin synthesis) (X-factor)

Limitation: Algorithm does not differentiate *H. haemolyticus* from *H. influenzae*. *H. haemolyticus* is rare and not a pathogen, so some over-reporting of *H. influenzae* may result. If growth takes longer than 24 h, may be *Francisella* which will not satellite, but is ALA-positive.

Francisella

1. Does not satellite,

2. ALA-negative.
3. Grows on CHOC but not on BAP.
4. Oxidase-negative; catalase weak.
5. Cefinase positive or penicillin resistant

ALA test procedure

Principle: Colonies that are ALA test-positive are able to convert delta-aminolevulinic acid to protoporphyrin and then to porphyrin which fluoresces red under ultraviolet light.

Reagent: Add 33.5 mg delta-aminolevulinic acid (Sigma Chemical Co., St. Louis, MO) and 20 mg MgSO₄·7H₂O to 100 mL of 0.1 M Sorensen's buffer, pH 6.9. (Appendix). Dispense in single use amounts and store at -20°C.

Procedure: Rub separate colonies onto filter paper in a clean petri dish. Pour the reagent over the colonies and incubate petri dish at 35°C for 2 h.

Interpretation: Observe reactions for red fluorescence (+) under long-wave ultraviolet light.

E. Identification requirements for *Moraxella catarrhalis*

1. Gram negative diplococci
2. Growth on blood agar plate as well as chocolate agar
3. Oxidase positive
4. Positive tributyrin test or butyrate disk

Limitation: Most *Moraxella* are butyrate-positive, but only *M. catarrhalis* is a diplococci.

Butyrate disk test

Disks are impregnated with bromo-chloro-indolyl butyrate as a substrate for the detection of butyrate esterase. The hydrolysis yields a blue colored compound after 5 minutes.

May also use indoxyl acetate disk – method, timing and blue color result is the same.

Reference: Speelefeld, E., J.M. Fossépré, B. Gordts, and H.W. Van Landuyt. 1994. Comparison of three rapid methods, tributyrine, 4-methylumbelliferyl butyrate, and indoxyl acetate, for rapid identification of *Moraxella catarrhalis*. J. Clin. Microbiol. 32: 1362-1363.

F. Identification of *Campylobacter jejuni*

1. Requires microaerobic environment
 2. Oxidase - positive
 3. Catalase - positive
 4. Curved rod
 5. Hippurate – positive
 - If hippurate - negative; indoxyl acetate -positive and cefazolin R identifies Nalidixic acid or ciprofloxacin R + cefazolin R + indoxyl acetate-negative is *C. lari*.
- Ciprofloxacin disks are no longer helpful for *C. jejuni* identification due to common resistance.

Rapid Hippurate

Hippurate test.

Principle: If organism is able to hydrolyse hippurate, it is determined by detecting glycine as a by-product of the hydrolysis.

Reagent: Prepare 2% aqueous sodium hippurate (Sigma) in distilled water. The solution can be aliquoted for individual use and stored frozen.

Procedure: Emulsify the organism in a small tube with 3 drops of reagent. Incubate for 2 h at 35°C. After incubation, add 2 drops of ninhydrin. A purple color indicates the hydrolysis of hippurate.

This test can be used to confirm the identification of *Streptococcus agalactiae*, *Campylobacter jejuni* and *Listeria monocytogenes*.

Note: Hippurate-negative *C. jejuni* can be separated from *C. lari* and *C. fetus* with indoxyl acetate. *C. jejuni* is positive and the latter two are negative in this test.

In Summary for Gram negative rods

- If growing on MAC, do
 1. Indole
 2. Oxidase
 3. Kit if not *E. coli*, *Proteus* or *Pseudomonas*
- If not growing on MAC, do
 1. Catalase
 2. Oxidase
 3. Gram stain
 4. Generally kits are not helpful here

III. Identification of Gram-positive cocci.

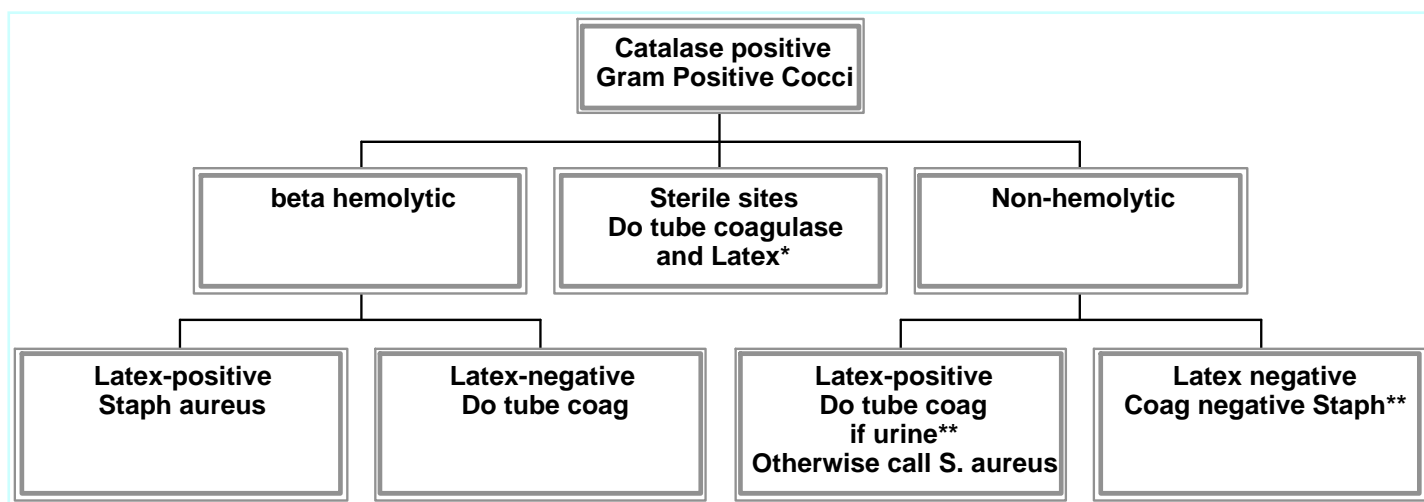
A. Identification requirements for *Staphylococcus aureus*

1. Grows on blood and chocolate agar as opaque, creamy, white, or light gold, convex colonies. (Some strains produce beta-hemolytic zones around the colonies).
2. Catalase-positive.
3. Gram-positive cocci in clusters.
4. Slide or tube (4 hr) coagulase-positive with rabbit plasma or positive Latex agglutination test.

Limitations:

1. **Latex tests** require less organism and are more rapid but have false-positives with *S. saprophyticus* and false-negatives with some methicillin-resistant *Staphylococcus aureus*. Confirm positive results of non-hemolytic colonies from urine specimens.
2. Because *S. lugdunensis* and *S. schleiferi* may be slide coagulase and Latex-positive, but tube-negative, it is recommended that both tests be performed on isolates from significant sterile site specimens.
3. The tube test must be read hourly up to 4 h if at 35°C, where the reaction is faster but the clot can lyse. Some staphylococci may require more than 4 hr for a positive test; negative tests at 4 h should be incubated at 25°C for a full 24 h.
4. Use only EDTA rabbit blood for test.

Figure 2. Identification algorithm for *Staphylococcus aureus* using Latex testing



* Do PYR if sterile site and tube coagulase-negative to rule out *S. lugdunensis*

** Do Novobiocin disk if urine and either Latex negative or tube coagulase-negative.

Staphylococcus lugdunensis

1. *Slide or Latex* coagulase variable
2. Tube coagulase-negative
3. PYR positive
4. Ornithine positive
5. Polymyxin B resistant
6. Use *S. aureus* breakpoints for oxacillin

Table 5. Biochemical reactions of the Staphylococcus species.

	Slide Coag	Latex agglu	Tube Coag	Ornithine Decarbox	Poly B	PYR
<i>S. aureus</i>	V	+	+	-	R	-?
<i>S. intermedius (dogs)</i>	V	V	+	-	S	+
<i>S. lugdunensis</i>	+	+	-	+	R	+
<i>S. schleiferi</i>	+	+	-	-	S	+
<i>S. saprophyticus</i> *	-	V	-	-	S	-
<i>S. epidermidis</i>	-	-	-	V	R	-
most other coagulase negative	-	-	-	-	S	V

*Novobiocin resistant.

B. Identification requirements for *Streptococcus pneumoniae*.

1. Gram stain with lancet-shaped gram-positive cocci in pairs or short chains
2. Catalase-negative
3. Colonies which are transparent, , slightly mucoid or flattened, and alpha hemolytic.
4. Bile soluble or Quellung-positive

Bile solubility test

Place a drop of 2% or 10% desoxycholate onto colonies of the suspected organism on a blood agar plate. After 30 min incubation at 35°C, colonies of *S. pneumoniae* will disintegrate. The test is based on the principle that *S. pneumoniae* produces an autolytic, intracellular enzyme that causes the organism to undergo rapid autolysis. There is also a slide and tube version of this test.

Limitation: Some *S. pneumoniae* are not bile-soluble and need optochin.

C. Identification requirements for *Enterococcus*.

1. Catalase-negative,
2. Gram-positive spherical cells, a bit elongated, arranged in pairs and chains.
3. Colonies are large > 1mm, non beta-hemolytic
4. PYR positive.

Limitation: Other gram-positive cocci can be PYR positive. *Aerococcus* is in tetrads and LAP-negative. *Globicatella* and *Dolosicoccus* colonies are tiny; they are LAP-negative. LAP is another rapid test just like PYR that uses same developer and same method. *Vagococcus* is motile. Motility can be easily done by incubating a 0.5 ml broth culture of the organism at 30°C for 30 minutes. *Lactococcus*, can be misidentified as *Enterococcus*; it is rare.

Table 6. Biochemical reactions of the PYR-positive cocci excluding *Streptococcus pyogenes*

Genus	Gram Stain	CAT	LAP	NaCl	10°C	45°C	Colony on BAP	Hemolysis	Bile esculin
<i>Enterococcus</i> (some motile)	ch	—	+	+	+	+	Large	α/γ/β	+
<i>Lactococcus</i>	ch	—	+	v	+	-	Large	α/γ	+
<i>Vagococcus</i> (motile)	ch	—	+	+	+	-	Large	α/γ	+
<i>Abiotrophia</i> / <i>Granulicatella</i>	ch	—	+	-	-	-	Satellite	α/γ	-
<i>Globicatella</i>	ch	—	-	+	-	-	Small	α	V
<i>Dolosicoccus</i>	ch	—	-	-	-	-	Small	α	NA
<i>Aerococcus viridans</i>	cl/t	—,w	-	+	-	-	Large	α	V
<i>Helcococcus kunzii</i>	cl/t	—	-	V	-	-	Tiny	γ	—
<i>Gemella</i>	cl/t/ch	—	V	-	-	-	Tiny, 48 h to grow	α/γ	—
<i>Facklamia</i> (hippurate +)	cl/ch	—	+	+	-	-	Small	γ	—
<i>Alloiococcus otitidis</i>	cl/t	w, +	+	+	-	-	Tiny, 72 h to grow	α	NA
<i>Ignavigranum</i> (hippurate -)	cl/ch	—	+	+	-	-	Satellite (v) or small	γ	—
<i>Rothia mucilaginosa</i>	cl	—,w, +	+	-	NA	NA	Sticky	γ	V
<i>Dolosigranulum</i>	cl/t	—	+	+	-	-	Small, rare	γ	NA

^a v, variable; cell arrangement in Gram stain: cl, clusters; t, tetrads; ch, chains.

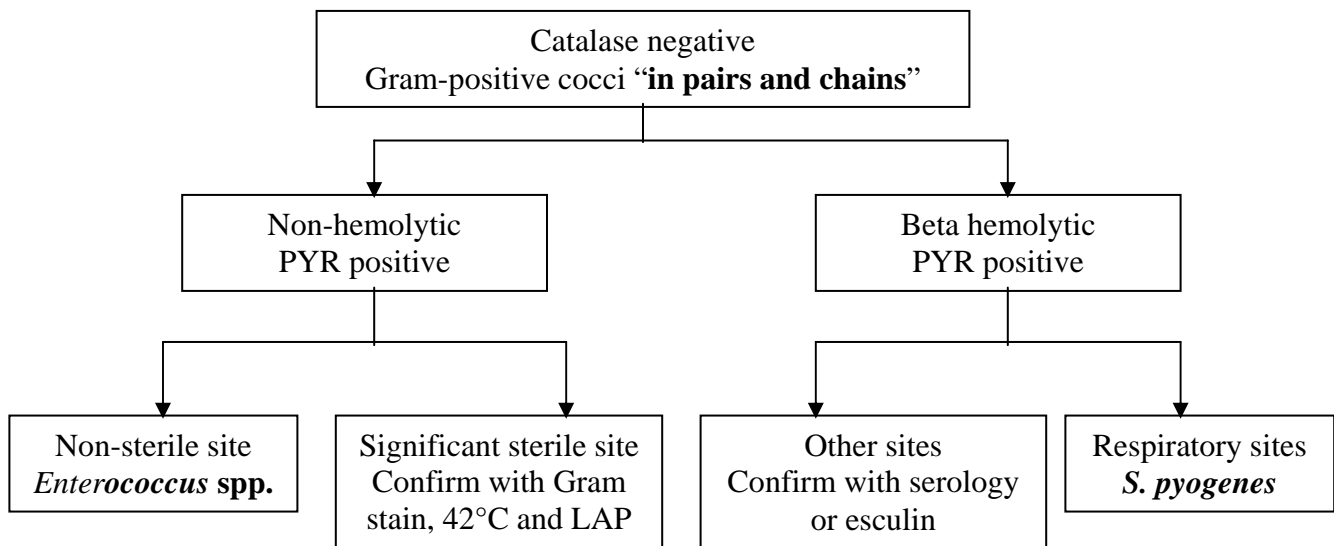
Table in part from LaClaire and Facklam 2000 J. Clin. Microbiol. June 38:2037-42.

D. Identification requirements for *Streptococcus pyogenes*

1. Catalase-negative
2. Gram-positive in pairs and chains.
3. Colonies are dry, peaked or convex and beta-hemolytic >0.5 mm in diameter after 24 h
4. PYR positive or particle agglutination.

Limitation: Enterococcus can be beta-hemolytic. Careful examination of colonies is necessary. . Large (>1 mm), moist colonies with a softer periphery of the zone of beta hemolysis should be tested for esculin. Enterococci are positive and *S. pyogenes* is negative for esculin. Errors would be very rare if test is only done on throat specimens.

Figure 3. Flow chart for identification of PYR-positive cocci



E. Identification requirements for *Streptococcus agalactiae* (Group B)

1. Catalase-negative: The importance of a negative catalase reaction in the identification of group B streptococci can not be overemphasized. *Listeria* have similar colony morphology.
2. Gram-positive cocci in pairs and chains.
3. Colonies are small, flat, semi-opaque, moist, with a small, soft zone or no zone of hemolysis
4. Positive in 30 minute CAMP-test or 2 h hippurate-test or particle agglutination confirms identification.

Limitation: Enterococcus can be hippurate-positive, but are PYR-positive; alpha streptococci can also be hippurate-positive. Therefore do not identify non-hemolytic colonies with hippurate.

CAMP test.

Principle: *S. agalactiae* produce a diffusable, extracellular protein that acts synergistically with staphylococcal beta lysin to lyse sheep cell erythrocytes. The beta lysin can be detected by enhanced zone of hemolysis around a disk containing beta lysin. The test is not rapid but requires overnight incubation. However, a rapid test can be performed by preparing beta lysin from a culture of *S. aureus*.

Reagent: Incubate a 10 ml broth culture of *S. aureus* ATCC 25923 for 24 h at 35°C. Filter sterilize the broth.

Procedure: Add a drop of the broth next to a colony of streptococcus. Incubate the plate at 35°C for 20 min. and observe for an arch of hemolysis much like that seen with the disk method.

III. YEAST IDENTIFICATION

A. Identification requirements for *Candida albicans*:

Oval budding yeast (on wet smear) having one of the following...

1. **Colonies with feet** described as colonies that exhibit mycelial projections into blood containing agar within the first 48hrs, called "star-like" or "feet" or
2. **Microscopic wet mount of yeast in calf serum demonstrating germ tubes**
The colony is incubated in fetal or newborn calf serum (not diluted) for a maximum of 3 h and observed microscopically for germ tubes. Presence of thin tube-like structures half the width of the yeast cell with no constriction is indicative of a positive test. About 5 % of *C. albicans* do not form germ tubes or
3. **Positive in 2 enzyme reactions in rapid kit:**
C. albicans possess two enzymes that make this species unique among the *Candida* species: L-proline aminopeptidase and beta-galactosaminidase. For the isolate to be *C. albicans* both reactions must be positive. Other yeast possess only one or neither enzyme. Kits with substrates to detect these enzymes are available from several manufacturers. Positive reactions are observed either colorimetrically or by fluorescence (as in the MUG test). The tests require a 5 min incubation and are more rapid than a 2 h germ tube test. Testing can only be performed from antibiotic-free media.

Limitation: *C. dubliniensis* can not be distinguished from *C. albicans* by any of these tests except by growth of the latter at 45°C. *C. tropicalis* can form fringe mistaken for feet, if incubation is extended.

B. Identification requirements for *Candida glabrata*

1. Colonies are small on blood or chocolate agar.
2. In a wet mount, the yeast cells are tiny without pseudohyphae.
3. Colonies are larger and more luxuriant on EMB than on blood agar at both 24h and 48 h **or**
Colonies are positive in RAT test incubated for 3 h at 42°C.

The RAT test for identification of *C. glabrata*

Principle: The Rapid Trehalose assimilation test is based on the unique characteristic of *C. glabrata* that it assimilates trehalose rapidly at 42°C

Reagent: RAT broth. See NCCLS document or purchase.

Procedure: Emulsify a colony in the broth and incubate it for 3 h at 42°C. A yellow color indicates a positive reaction.

Limitation: Although a few strains of *C. tropicalis* can also assimilate trehalose, they usually do not do it within 3 h. If they do, they can be separated from *C. glabrata* by colony morphology, yeast size and presence of pseudohyphae in wet mount.

C. Identification requirements for *Cryptococcus neoformans*

- 1) Colonies are large and non-pigmented on blood agar, but may be mucoid.
- 2) On wet mount, spherical to round cells of variable size; budding can be present but hyphae are not.
- 3) A capsule may be seen by India ink prep.
- 4) Positive caffeic acid test within 4 h at 35°C.

C. neoformans is unique in that it possesses phenol oxidase. This enzyme is detected by its ability to produce melanin from caffeic acid and ferric citrate. The result is a brown pigment which is absorbed by the cell wall. The test must be done from medium without dextrose.

Note: *Cryptococcus* are also positive in the rapid urea reaction within 30 min. of inoculation. The rapid urea test can be useful in the identification of *Proteus*, *Helicobacter pylori*, *Brucella*, and *Bordetella* species.

IV. Abbreviated Identification of Anaerobic Gram-Negative Bacteria (use 15% H₂O₂ for catalase)

Identification	Blood/LKV Colony Morphology	Cell Morphology	Bacteroides Bile Esculin Colony Morphology	Indole Reaction
<i>Bacteroides fragilis</i> group	Large, convex	Regular	Large, convex, gray-black	Not Done
<i>Bacteroides ureolyticus</i>	Translucent, pitting the agar; catalase-negative urea-positive	Tiny rods, coccobacilli	No growth	Negative
<i>Bilophila wadsworthia</i>	Tiny, translucent (catalase +++)	Regular to filaments	Translucent with black center at 72 h	Negative
<i>Fusobacterium nucleatum</i>	Opalescent, breadcrumb	Fusiform, thin pointed	No growth	Positive
<i>Porphyromonas</i> spp.	Small, translucent or opaque, fluoresce brick-red on Brucella agar	Tiny coccobacilli	No growth	Positive
<i>Prevotella intermedia</i>	Small, translucent or opaque, fluoresce brick-red on LKV or Brucella agar	Tiny coccobacilli	No growth	Positive
<i>Prevotella</i> spp.				Negative
<i>Veillonella</i> spp.	Small, transparent or opaque, fluoresce brick-red on Brucella agar	Tiny diplococci	No growth	Negative

Abbreviated Identification of Anaerobic Gram-Positive Bacteria

Identification	Blood Agar Colony Morphology	Cell Morphology	Indole Reaction
<i>C. difficile</i>	Large, flat colonies; barnyard (cow manure) smell; chartreuse fluorescence on Brucella, catalase-negative	Thin rods, rare spores	Negative
<i>C. perfringens</i>	Large, irregular-shaped, double zone beta-hemolysis; catalase-negative	Boxcar, large, square rods	Not Done
<i>C. septicum</i>	Smoothly swarming; catalase-negative	Thin rods, subterminal spores	Negative
<i>C. sordellii</i>	Very large, lobate, irregular, flat, catalase-negative; urea-positive	Thin rods, subterminal spores	Positive
<i>C. tetani</i>	Smoothly swarming but slow growing	Swollen terminal spores	Positive
<i>Peptostreptococcus</i> spp.*	Small, peaked, circular	Cocci, pairs and chains	Not Done
<i>Propionibacterium acnes</i>	Small, opaque, enamel-white, circular (catalase + with 15% H₂O₂)	Coryneform rods	Positive

* May be newly named genera (*Peptoniphilus*, *Schleiferella*, *Anaerococcus*, *Finegoldia*, *Micromonas*). Until the new genera are better recognized, it is still prudent to report them as “*Peptostreptococcus* species” or “anaerobic gram positive cocci.”

SUMMARY OF RAPID TESTING FOR COMMON ORGANISMS

Organism	Initial Observations	Confirmatory Rapid Tests	Comments and Limitations
<i>Escherichia coli</i>	Typical gram-negative morphology on BAP without spreading	Spot indole-positive and oxidase-negative plus one of the following: 1. Hemolytic or 2. PYR-negative and lactose-positive 3. MUG-positive	Some <i>M morganii</i> and <i>P vulgaris</i> are hemolytic and indole-positive, but are lactose-negative; do not do MUG on lactose-negative colonies from abdominal sites
<i>Proteus mirabilis</i>	Spreading colony	Indole-negative	If ampicillin-resistant could also be <i>Proteus penneri</i> , which, unlike <i>P mirabilis</i> , is rapid maltose-positive
<i>Proteus vulgaris</i>	Spreading colony	Indole-positive	
<i>Pseudomonas aeruginosa</i>	Metallic, pearlescent, rough, pigmented or extremely mucoid colony Grape or tortilla odor	Oxidase-positive	Identification is definitive but should be confirmed as colistin-sensitive from cystic fibrosis patients
<i>Haemophilus influenzae</i>	Gram-negative coccobacilli showing no growth on blood agar but growth on chocolate in 24 h	ALA-negative	Need hemolysis on horse or rabbit blood to separate from <i>H haemolyticus</i>
<i>Moraxella catarrhalis</i>	Gram-negative diplococcus growing both on blood and chocolate agar	Oxidase-positive and Butyrate esterase-positive	
<i>Enterococcus</i>	Non-hemolytic, gram-positive cocci in chains	Catalase-negative and PYR-positive	Cannot rapidly separate from rare <i>Lactococcus</i> , which does not grow at 42°C.
<i>Streptococcus aureus</i>	White, cream to yellow opaque colonies, that are generally hemolytic; gram-positive cocci in clusters	Catalase-positive, Slide coagulase or 4 h tube coagulase-positive or latex agglutination kit for staphylococci-positive	<i>S. lugdunensis</i> and <i>S. schleiferi</i> can be slide, but not tube, coagulase-positive. <i>S. saprophyticus</i> can be latex positive but is non-hemolytic and is a urinary isolate.
<i>Streptococcus agalactiae</i>	Gram-positive cocci in pairs and chains with a small zone of hemolysis	Catalase-negative and either rapid CAMP-positive in 30 min or rapid hippurate-positive in 2 h or positive serogrouping by particle agglutination	Enterococci can be hippurate-positive, beta-hemolytic; they are PYR-positive. Non-hemolytic <i>S. agalactiae</i> cannot be identified with hippurate.
<i>Streptococcus pneumoniae</i>	Gram-positive cocci in pairs and chains; α -hemolytic; transparent colonies on BAP	Catalase-negative and Bile-soluble (using 2 or 10% sodium deoxycholate) on BAP	Bile resistant, typical colonies should be confirmed by other methods

<i>Streptococcus pyogenes</i>	Gram-positive cocci in pairs and chains having β -hemolytic colonies >0.5 mm in diameter on BAP	Catalase-negative and PYR-positive or positive serogrouping by particle agglutination	β -hemolytic enterococci are PYR-positive but have larger colonies with a soft periphery of the zone of hemolysis.
<i>Candida albicans</i>	Smooth colony possibly with “feet”; large yeast cells	Feet is definitive or germ tube-positive in <3 h, if feet are not present or positive for rapid enzyme for PRO and BGAL or apple green colonies at 48 h on chrom agar	Use fetal or newborn calf serum. Will not separate from <i>C. dubliniensis</i> .
<i>Candida glabrata</i>	Tiny colony on blood agar and tiny yeast in smear	Rapid assimilation of trehalose in 2 h at 42°C	If growth is better on EMB than BAP, trehalose test is not needed
<i>Cryptococcus neoformans</i>	Large and non-pigmented colonies on blood agar, may be mucoid. Spherical to round cells of variable size; budding can be present but hyphae are not. Capsule observed with India Ink	Urease-positive and positive for caffeic acid within 4 h at 35°C	

ALA = delta-aminolevulinic acid; BAP = sheep blood agar plate; EMB = eosin methylene blue; MAC = MacConkey; MUG = 4-methylumbelliferyl- β -D-glucuronide; PYR = L-pyrrolidonyl β -naphthylamide, PRO = L-proline- β -naphthylamide, BGAL = either N-acetyl- β -D-galactosamide or methylumbelliferyl-N-acetyl- β -D-galactosaminide

Appendix Sorensen's buffer

1. Solution A: 7.1 g Na_2HPO_4 (anhydrous salt previously dried at 130°C) in 500 mL deionized water.
2. Solution B: 6.8 g KH_2PO_4 (anhydrous salt previously dried at 110°C) in 500 mL deionized water.
3. When making solutions, check pH; adjust with solution A to make more alkaline; solution B to acidify).
4. 0.1 M, pH 6.9: Combine 55.4 ml of solution A and 44.6 ml of solution B.
5. M/15 buffer, pH 7.5: Combine 56.7 mL of solution A, 10 mL of solution B, and 33.3 ml deionized water.

References:

Exhaustive list is in NCCLS document.